

AMENDMENTS TO THE SPECIFICATION

Amend the title of the invention as follows.

~~METHOD OF INHIBITING~~ FOR SUPPRESSING TUMOR PROLIFERATION

Insert the following paragraph immediately beneath the title at page 1 of the English language specification.

CROSS-REFERENCE TO REPLATED APPLICATIONS

The present application is the U.S. National Stage of PCT/JP2005/004485, filed March 15, 2005, which, in turn, claim the benefit of Japanese Patent Application Serial No. JP 2004-074570, filed March 16, 2004.

Amend the paragraph beginning at page 2, line 7, of the English language specification as follows.

Separately from this, the present inventors recently proved that expression of a polypeptide involved in angiogenesis in mesenchymal cells (MCs), but not in endothelial cells (ECs), plays an essential role in therapeutic angiogenesis for the therapy of severe limb ischemia using fibroblast growth factor-2 (FGF-2)(Masaki I *et al.*, Circ Res. 90: 966-973 (2002); Onimaru M *et al.*, Circ Res. 91: ~~723-730~~ 923-930 (2002)). FGF-2

stimulates local expression of VEGF and another angiogenic growth factor, hepatocyte growth factor/scatter factor (HGF/SF), in vascular mesenchymal cells (MCs: including pericytes, vascular smooth muscle cells, and adventitial fibroblasts) (Onimaru M *et al.*, Circ Res. 91: ~~723-730~~ 923-930 (2002)). Interestingly, time courses of FGF-2-mediated HGF/SF expression are biphasic, meaning that upregulation in the early phase does not require new protein synthesis, but that upregulation in the late phase is mediated and sustained by the endogenous platelet-derived growth factor receptor- α (PDGFR α)-p70S6 kinase pathway (Onimaru M *et al.*, Circ Res. 91: ~~723-730~~ 923-930 (2002)).

Amend the paragraph beginning at page 3, line 5, of the English language specification as follows.

The biological role of PDGFR α has long been the subject of argument. PDGF-A homodimers (PDGF-AA) induce the DNA synthesis and proliferation of NIH3T3 cells. On the other hand, however, in other cells they inhibit chemotaxis reactions induced by other reagents (Siegbahn A *et al.*, J Clin Invest. 85: 916-920 (1990)). While there is little evidence of PDGF receptor expression in endothelial cells, PDGF receptor ligands, including not only PDGF-AA and PDGF-BB but also the novel PDGF, PDGF-CC (Li X *et al.*, Nat Cell Biol. 2: 302-309(2000), stimulate angiogenesis *in vivo* (Nicosia RF *et al.*, Am J Pathol. 145: 1023-1029 (1994); Cao R *et al.*, FASEB J. 16: 1575-1583 (2002)). These findings suggest the possibility that other angiogenesis-stimulating factors also

mediate the PDGF-dependent angiogenesis process. In line with previous studies (Onimaru M *et al.*, Circ Res. 91: ~~723-730~~ 923-930 (2002)), the present invention suggests that the PDGFR α system is essential for sustaining the angiogenesis signals that use VEGF and HGF/SF in MCs. However, since all of these ligands activate PDGFR α and each can cause different cellular responses, the essential ligands for angiogenesis have not been determined. The present invention shows that of the PDGFR α ligands, PDGF-A in particular plays an important role in the formation of tumor vasculature. Since enhanced PDGF-A expression closely relates to tumor malignancy, tumor proliferation was dramatically suppressed upon inhibiting PDGF-A expression in tumor cells (Example 5). Thus, the present invention clarifies that inhibition of PDGF-A expression or inhibition of the binding between PDGF-AA and PDGFR α can result in efficient suppression of tumor angiogenesis, thereby bringing about tumor dormancy.

Amend the paragraph beginning at page 25, line 30, of the English language specification as follows.

Doses of the antitumor agents described herein may vary depending on patient body weight, age, sex and symptoms, the form of the composition to be administered, the administration methods, and so on, and doses can be appropriately determined by those skilled in the art. The frequency of administration is one or more times, within the range of clinically acceptable side effects. Administration may also be to one or more sites.

When administered orally, adult doses of non-peptide low molecular weight compounds are generally within the range of about 0.1 to 100 mg per day, preferably about 1.0 to 50 mg per day, and more preferably about 1.0 to 20 mg per day (for 60 kg in body weight). When administered parenterally, doses vary depending on the subject to be administered, the target organ, symptoms, and administration route, but doses can be injected intravenously when administered in injectable forms, and range from, for example, about 0.01 to 30 mg per day, preferably about 0.1 to 20 mg per day, and more preferably about 0.1 to 10 mg per day. For other animals, for example, the doses can be calculated by correcting the above doses for weight. The doses of protein formulations will range from about 100 μ g to 50 mg per day, for example. For example, the administration site for viral vectors may be one or more sites (for example, two to ten sites) inside or in the vicinity of the tumor. Preferable doses of ~~adenoviruses~~ adenoviral vectors are, for example, 10^{10} to 10^{13} pfu, more preferably 10^{11} to 10^{13} pfu. The preferable doses of minus strand RNA ~~viruses~~ virus vectors are, for example, 2×10^5 CIU to 5×10^{11} CIU. The administration sites for naked DNAs, antisense nucleic acids, siRNAs, or such, may be one or more sites (for example, two to ten sites) inside or in the vicinity of the tumor. Preferable doses per site are, for example, 10 μ g to 10 mg, and more preferably 100 μ g to 1 mg. When vector-introduced cells are administered *ex vivo*, for example, the viral vectors are introduced into target cells outside the body (for example, in test tubes or in dishes) at an MOI of one to 500. The transgenic cells can be transplanted into tumors at

doses of 10^5 to 10^9 cells, and preferably 10^6 to 10^8 cells. The document Freedman SB *et al* Ann Intern Med, 136: 54-71 (2002) can be referred to regarding doses. Animal subjects for the treatments include humans and other desired non-human animals, specifically humans, monkeys, mice, rats, rabbits, sheep, cattle, and dogs.

Amend the paragraph beginning at page 27, line 16, of the English language specification as follows.

HSMC (J. Cell Biol., 50: 172-86 (1971)), MRC-5 (ATCC CCL-171), SAS (J. Biol. Chem., 270 (41): 24321-69 (1995)), MH134 (J. Natl. Cancer Inst., 17: 1-21 (1956)), QG56 (Int. J. Cancer, 35 (6): 808-12 (1985)), TF (Cancer, 69 (10): 2589-97 (1992)), KN (Cancer, 69 (10): 2589-97 (1992)), EBC-1 (Am. J. Pathol., 142 (2): 425-31 (1993)), PC9 (Int. J. Cancer, 15 (4): 449-55 (1985)), and COS7 Cells (ATCC CRL-1651) were purchased from American Type Culture Collection (ATCC). As mentioned previously, the intracellular signal inhibitors below were each used at the following concentrations for HSMC and MRC5 cells (Onimaru M *et al.*, Circ Res. 91: ~~723-730~~ 923-930 (2002)): Ras, Ras-inhibitory peptide (50 μ mol/L, Alexis Japan, Tokyo, Japan); p70S6K, p70S6K inhibitor rapamycin (100 ng/ml, Sigma-Aldrich Japan, Tokyo, Japan); PKC, PKC inhibitor bisindolylmaleimide (100 nmol/L, Sigma); PI3K, PI3K-inhibitor wortmannin (120 nmol/L, Sigma); MEK inhibitor U0126 (10 μ mol/L, Promega K.K., Tokyo, Japan); PKA, ~~PKA-inhibitory~~ PKA-inhibitory peptide (1 μ mol/L, Calbiochem, San Diego, CA);

and NF- κ B, NF- κ B inhibitor ALLN (5 μ mol/L, Roche Diagnostics, Tokyo, Japan). Anti-PDGF-AA-neutralizing goat antibody, anti-PDGFR α neutralizing goat antibody, and control goat IgG were purchased from R&D systems (Minneapolis, MN). The stocks of recombinant SeVs, including mouse FGF-2-encoding SeV (SeV-FGF2) and firefly luciferase-encoding SeV (SeV-luciferase) used in the present invention were prepared as mentioned previously (Masaki I *et al.*, Circ Res. 90: 966-973 (2002); Onimaru M *et al.*, Circ Res. 91: ~~723-730~~ 923-930 (2002)). Recombinant SeV expressing the extracellular domain of human PDGFR α was constructed as follows: Total RNA was extracted from MRC-5 cells; cDNA was then synthesized from this total RNA by reverse transcription and used as a template to amplify cDNA fragments (amplified region: position 1-1575 bases of CDS) using synthetic primers with restriction enzyme site tags (forward-BglII: 5'-aaAGATCTatggggacttcccatccggc-3' (SEQ ID NO: 9) and reverse-NheI: 5'-ttGCTAGCtcactgtgtcatcgtcgtcctttagtcttcagaacgcagggt-3' (SEQ ID NO: 10); and the obtained cDNA fragments were subcloned into pcDNA3.1 (Invitrogen, Carlsbad, CA) (SEQ ID NOs: 7 and 8). Clones whose entire sequence was confirmed by capillary sequencer (model CEQ2000L, Beckman Coulter Inc., Fullerton, CA) to be completely identical to a reported known sequence (GenBank No. NM_006206) were subcloned into the template plasmid encoding SeV18+ (Hasan, M. K. *et al.*, J. Gen. Virol. 78: 2813-2820 (1997)). Recombinant SeV (SeV-hsPDGFR α) expressing soluble human PDGFR α was recovered, as mentioned previously (Masaki I *et al.*, Circ Res. 90: 966-973 (2002);

Onimaru M *et al.*, Circ Res. 91: ~~723-730~~ 923-930 (2002); Yonemitsu Y *et al.*, Nat Biotechnol. 18: 970-973 (2000)). Soluble human PDGFR α was confirmed by Western blotting to be secreted into the culture supernatant of COS7 cells to which SeV-hsPDGFR α had been introduced (data not shown).

Amend the paragraph beginning at page 28, line 24, of the English language specification as follows.

Details of surgical procedures and limb prognosis evaluation are described (Masaki I *et al.*, Circ Res. 90: 966-973 (2002); Onimaru M *et al.*, Circ Res. 91: ~~723-730~~ 923-930 (2002)). For gene transfer, 25 μ l of vector solution was injected into two portions of femoral muscle, immediately after the operation. Endogenous PDGF-AA activity was suppressed *in vivo* using PDGF-AA-specific neutralizing goat polyclonal IgG (cross-reactive to human and mouse proteins) (R&D) via a disposable micro-osmotic pump (Model 1007D, ALZA Co., Mountain View, CA), as described previously (Masaki I *et al.*, Circ Res. 90: 966-973 (2002); Onimaru M *et al.*, Circ Res. 91: ~~723-730~~ 923-930 (2002)).

Amend the paragraph beginning at page 29, line 4, of the English language specification as follows.

As mentioned previously (Masaki I *et al.*, Circ Res. 90: 966-973(2002); Onimaru

M *et al.*, Circ Res. 91: ~~723-730~~ 923-930 (2002)), the protein contents of the mouse limb muscle, tumor, and culture medium were determined using Quantikine Immunoassay systems for mice (both the 164 and 220 amino acid residue forms are recognized) and human VEGF-A, human FGF-2 (available to both human and mouse), human HGF (R&D Systems Inc., Minneapolis, MN), and rat HGF (available to mouse HGF; Institute of Immunology Inc., Tokyo, Japan), according to the manufacturer's instructions.

Amend the paragraph beginning at page 30, line 23, of the English language specification as follows.

As mentioned previously, blood flow in the tumors was assessed using a Laser Doppler perfusion image (LDPI) analyzer (Moor Instruments, Devon, UK) (Masaki I *et al.*, Circ Res. 90: 966-973 (2002); Onimaru M *et al.*, Circ Res. 91: ~~723-730~~ 923-930 (2002)). To remove background noise from blood flow in the small intestine, a blue-sheet was inserted into the peritoneal cavity immediately prior to assessment. To minimize data variables due to ambient light and temperature, the LDPI index was represented as the ratio of tumor pixels to scrotal pixels.

Amend the paragraph beginning at page 31, line 26, of the English language specification as follows.

In addition to the cooperative effect of FGF-2 and PDGF-AA on the expression of

VEGF and HGF/SF in MCs, the present inventors had previously discovered that FGF-2 enhances endogenous expression of PDGF-AA via Ras and p70S6K signal transductions, which contribute to the sustained expression of HGF/SF in HSMC (Onimaru M *et al.*, Circ Res. 91: ~~723-730~~ 923-930 (2002)). The present inventors hypothesized that an analogous system involving VEGF and MGF/SF expression also exists in fibroblasts (MRC5 cells). As seen in previous studies, FGF-2 typically upregulated the VEGF and HGF/SF proteins; and a MEK inhibitor, Ras-inhibitory peptide, and p70S6K inhibitor (RAPA) removed these effects (Fig. 2A). The repeated Northern blot analysis of time courses of FGF-2-mediated VEGF expression in MRC5 cells showed that biphasic (at three hours and after that) upregulation of VEGF occurs (Fig. 2B), as seen previously in HGF/SF expression using HSMC (Onimaru M *et al.*, Circ Res. 91: ~~723-730~~ 923-930 (2002)). Early phase VEGF expression was not affected by RAPA treatment, but RAPA treatment caused sustained expression in later phases to completely disappear (Fig. 2B). Moreover, FGF-2-mediated upregulation of VEGF protein was completely eliminated by an anti-PDGFR α antibody (Fig. 2C), as observed in RAPA treatment (Fig. 2A). Since the same result was obtained for HGF/SF expression (data not shown), it was concluded that the PDGFR α system plays a critical role in enhancing and sustaining FGF-2-mediated expression of VEGF and HGF/SF in MCs.

Amend the paragraph beginning at page 32, line 12, of the English language specification as follows.

In order to investigate the predictable cascade-like relationship of FGF-2, PDGFR α and VEGF/HGF *in vivo*, two separate mouse limb ischemia models, namely, C57BL/6 mouse limb salvage model and balb/c nu/nu mouse limb autoamputation model (Masaki I *et al.*, Circ Res. 90: 966-973 (2002)) were assessed *in vivo* using a recombinant Sendai virus (SeV-FGF2) that expresses FGF-2 (Masaki I *et al.*, Circ Res. 90: 966-973 (2002); Onimaru M *et al.*, Circ Res. 91: ~~723-730~~ 923-930 (2002); Compagni A *et al.*, Cancer Res. 60: 7163-7169 (2000); Yonemitsu Y *et al.*, Nat Biotechnol. 18: 970-973 (2000); Masaki I *et al.*, FASEB J. 15: 1294-1296 (2001); Yamashita A *et al.*, J Immunol. 168: 450-457 (2002); Shoji F *et al.*, Gene Ther. 10: 213-218 (2003)). FGF-2 overexpression was confirmed in the limb salvage model using ELISA assays (data not shown); however, upregulation of both PDGF-A and PDGFR α mRNA was confirmed by real-time quantitative PCR assays (Figs. 3A and 3B). In the same tissue samples, expression of VEGF and HGF/SF were similarly enhanced by FGF-2, and an anti-PDGF-AA neutralizing antibody eliminated this effect, as did RAPA treatment (Figs. 3C and 3D). The effect of RAPA was also confirmed at the protein level (Figs. 3E and 3F). Moreover, since the anti-PDGF-AA antibody and RAPA eliminated the therapeutic effect of FGF-2 in the limb autoamputation model (Fig. 4), the PDGFR α system was shown to also play a critical role in FGF-2-mediated therapeutic angiogenesis.